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(54) Volume measurement of microbial organisms

(57) A method and apparatus for cell volume and biomass estimation by sampling a fermentation broth and analyzing an image of the broth using brightness thresholds to distinguish between background, cellular regions and cytoplasmic and degenerated regions and summing corresponding areas in the image. Volumes can be calculated from the areas by treating the organisms as geometrical bodies and biomass derived by multiplying the volume by a density.

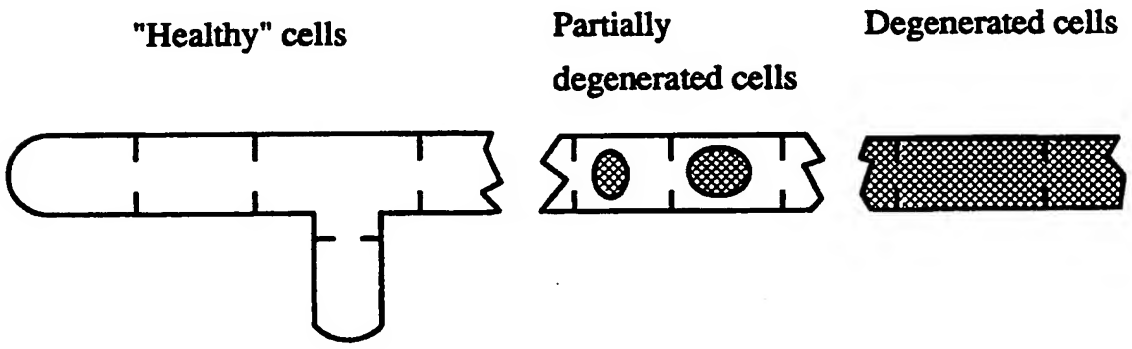


Fig. 1

GB 2 254 414 A

FIG 9.

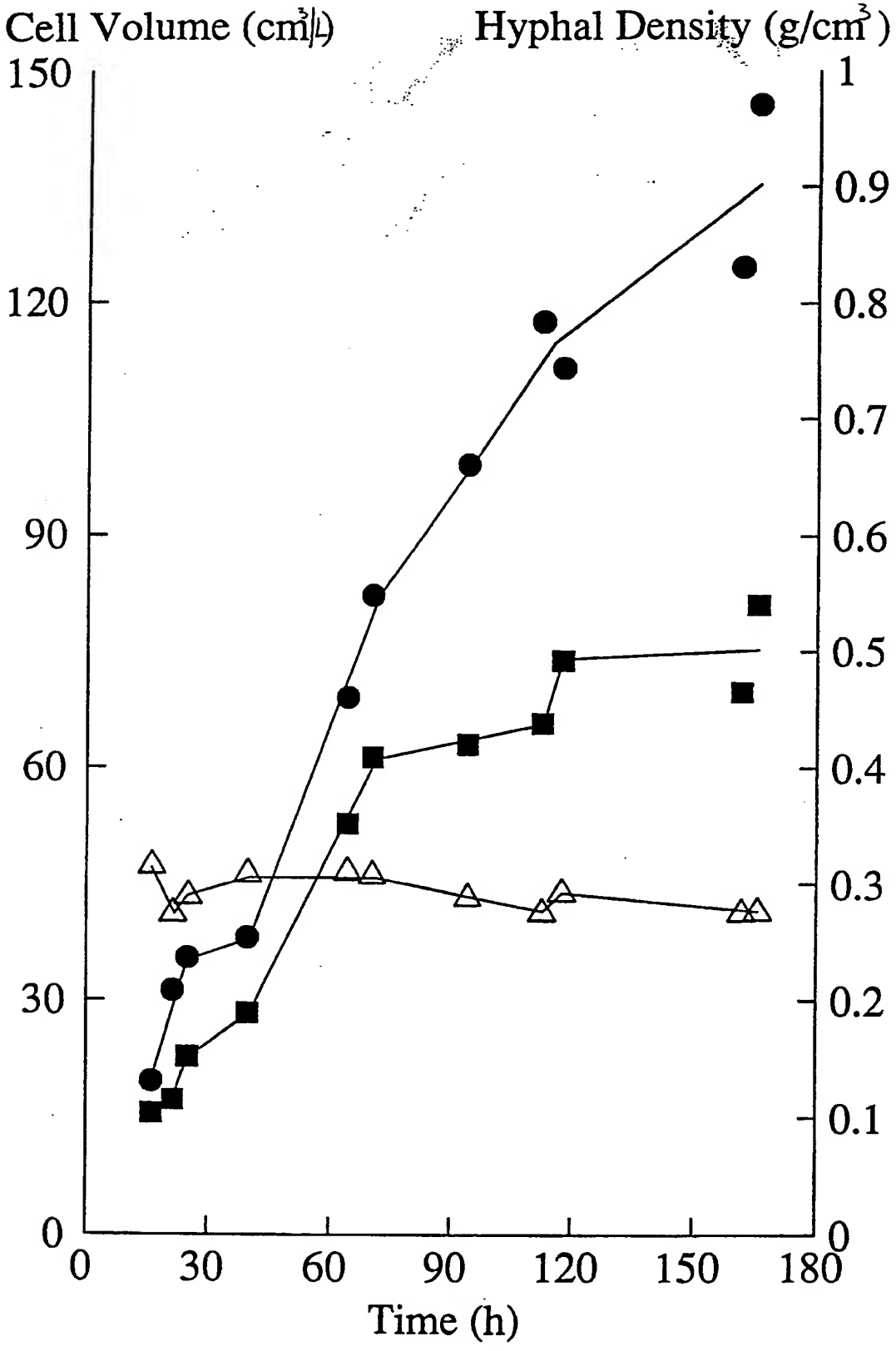
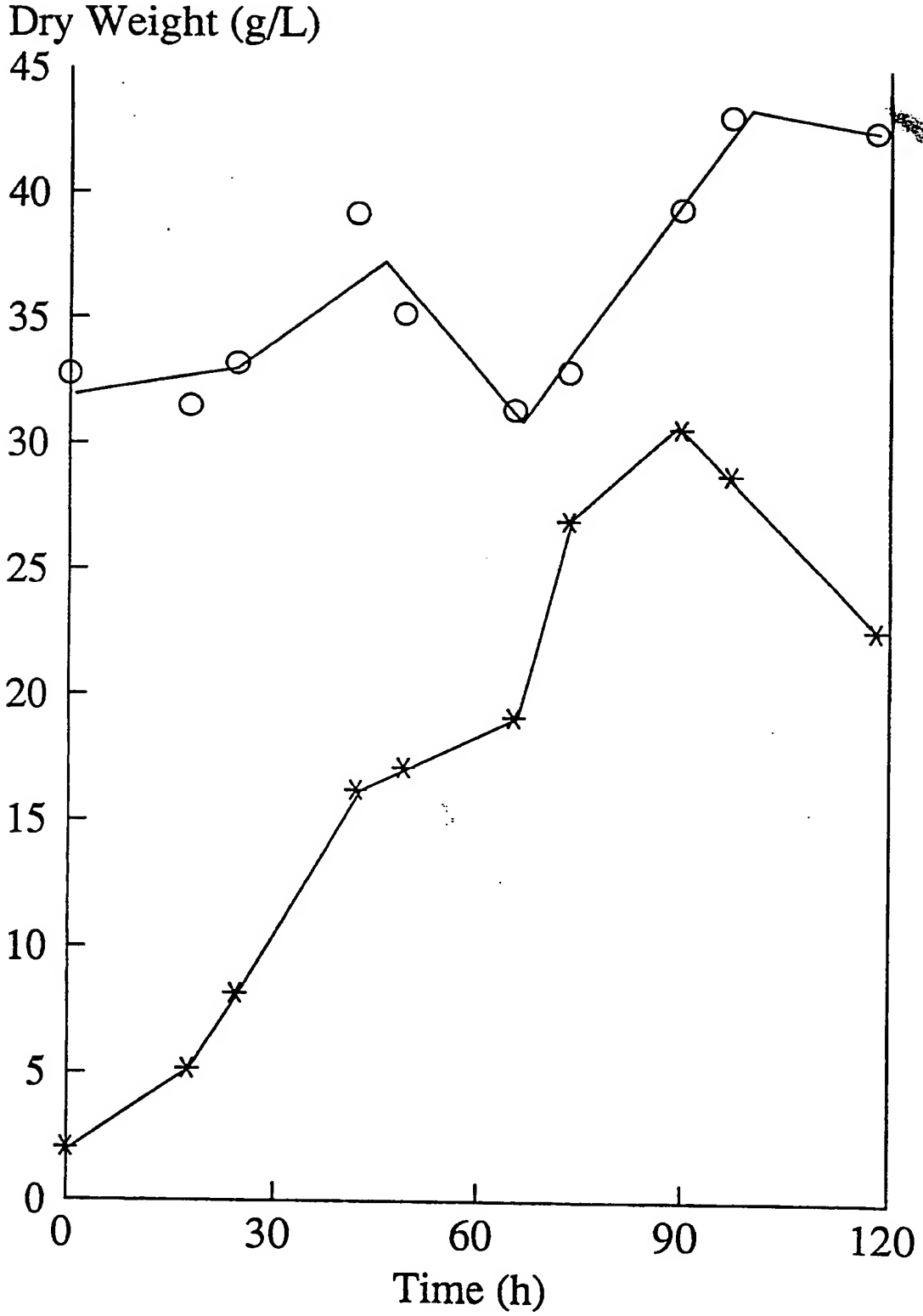

Cell Volume (cm³/L)
Hyphal Density (g/cm³)
150
120
90
60
30
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
30
60
90
120
150
180
Time (h)

FIG 10.



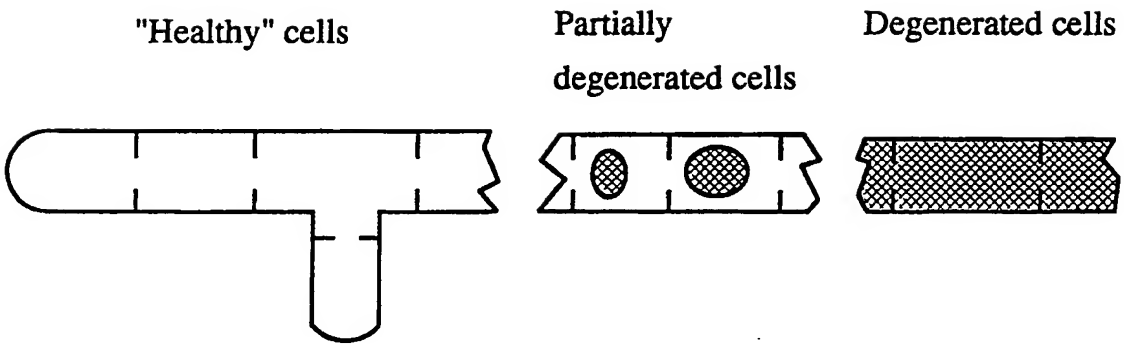

"Healthy" cells
Partially degenerated cells
Degenerated cells

FIG. 1

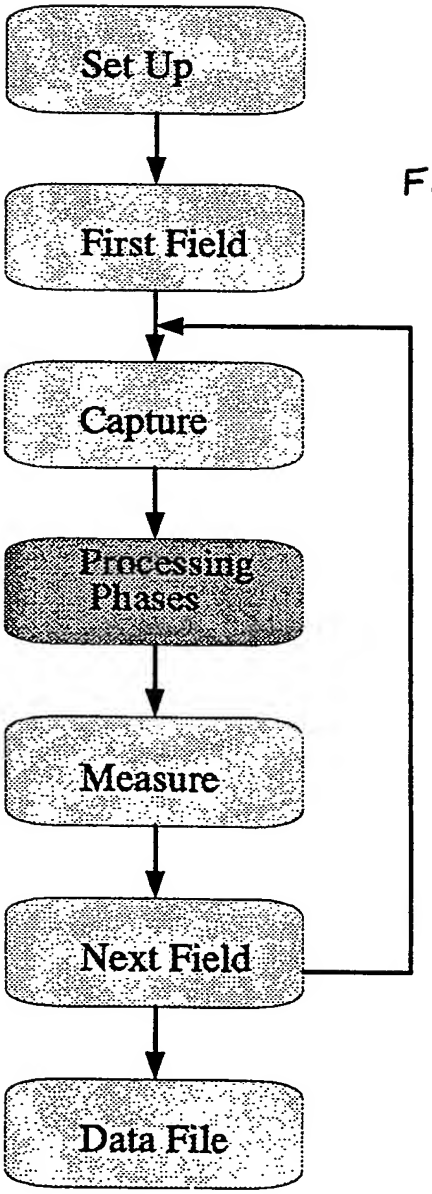

Set Up
First Field
Capture
Processing Phases
Measure
Next Field
Data File

FIG 2.

FIG 3A

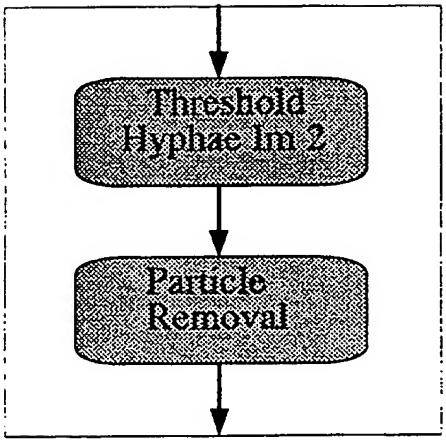


FIG 3B

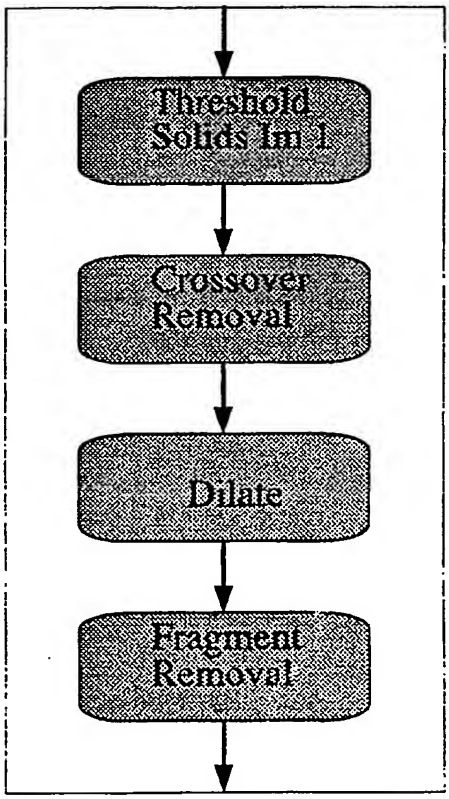
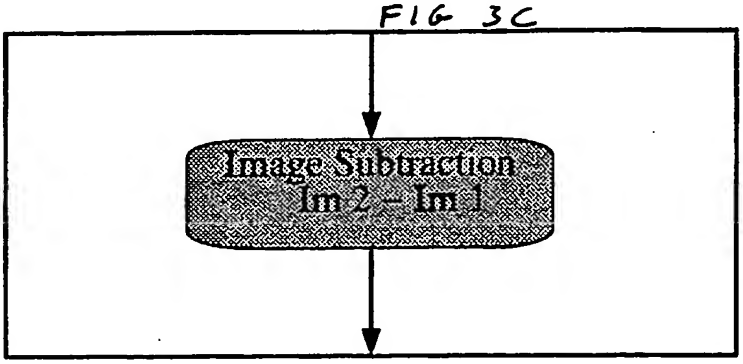


FIG 3C



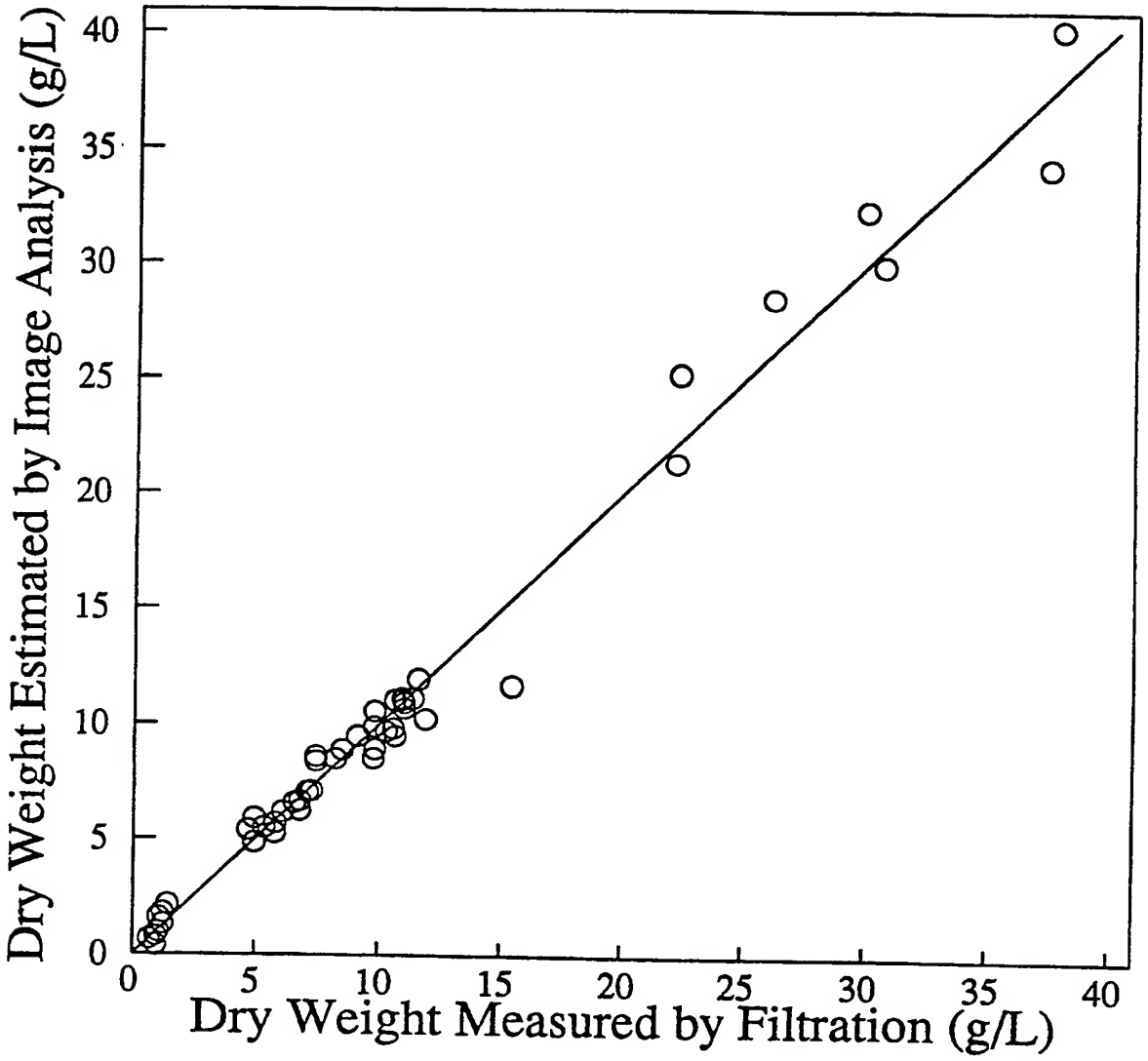

Dry Weight Estimated by Image Analysis (g/L)
Dry Weight Measured by Filtration (g/L)
0
5
10
15
20
25
30
35
40

FIG 4.

FIG 5.

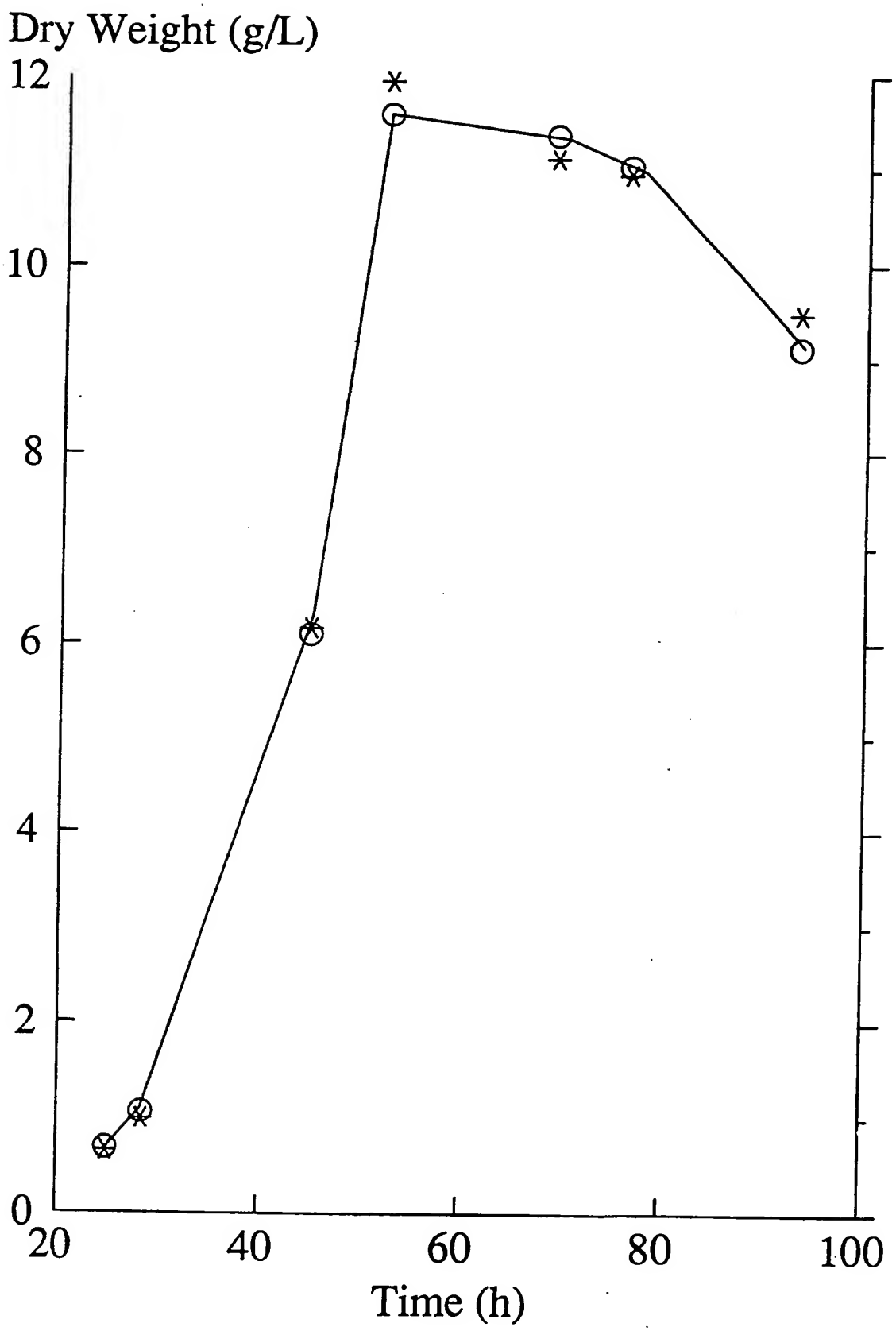


FIG 6.

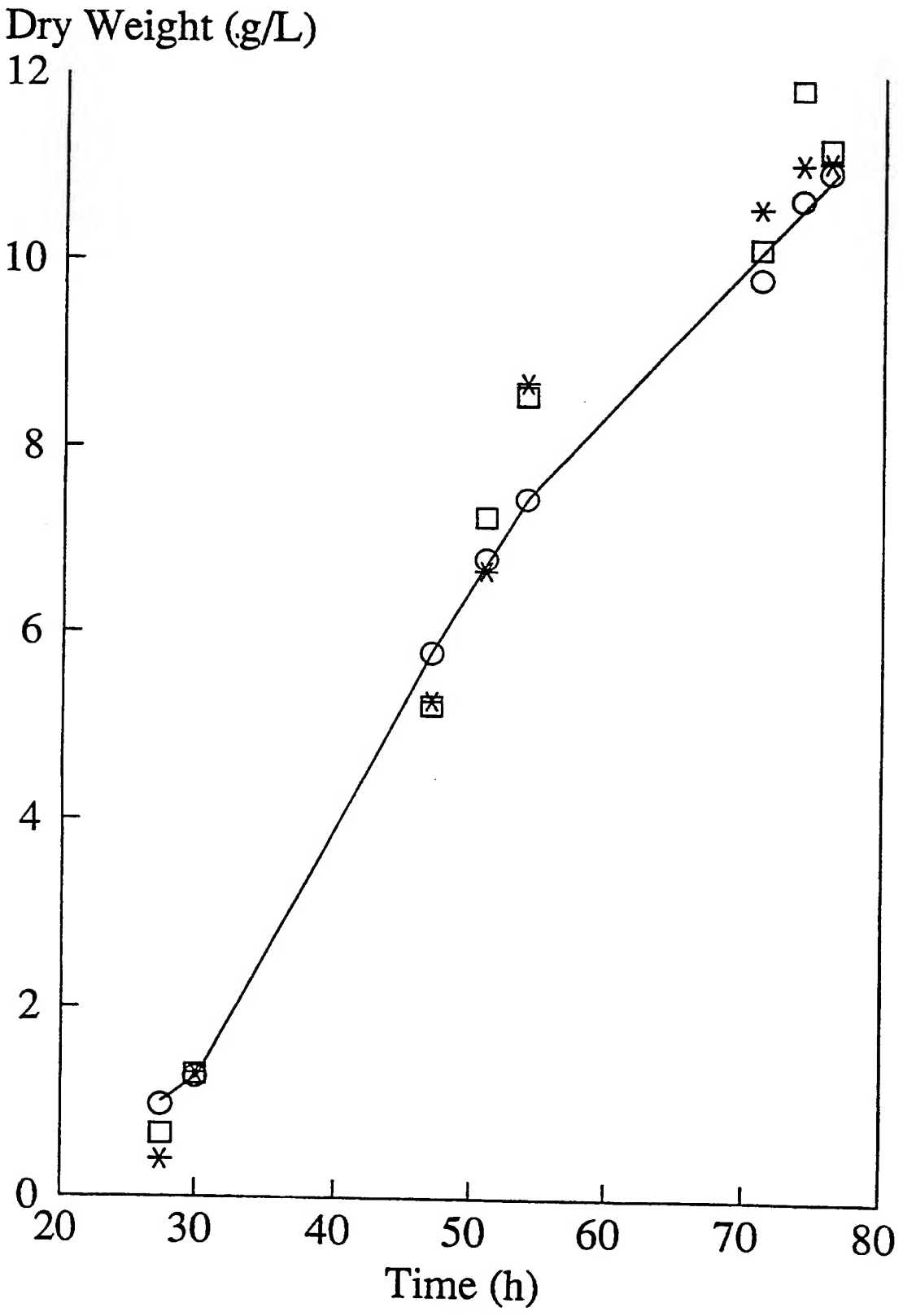


FIG 7

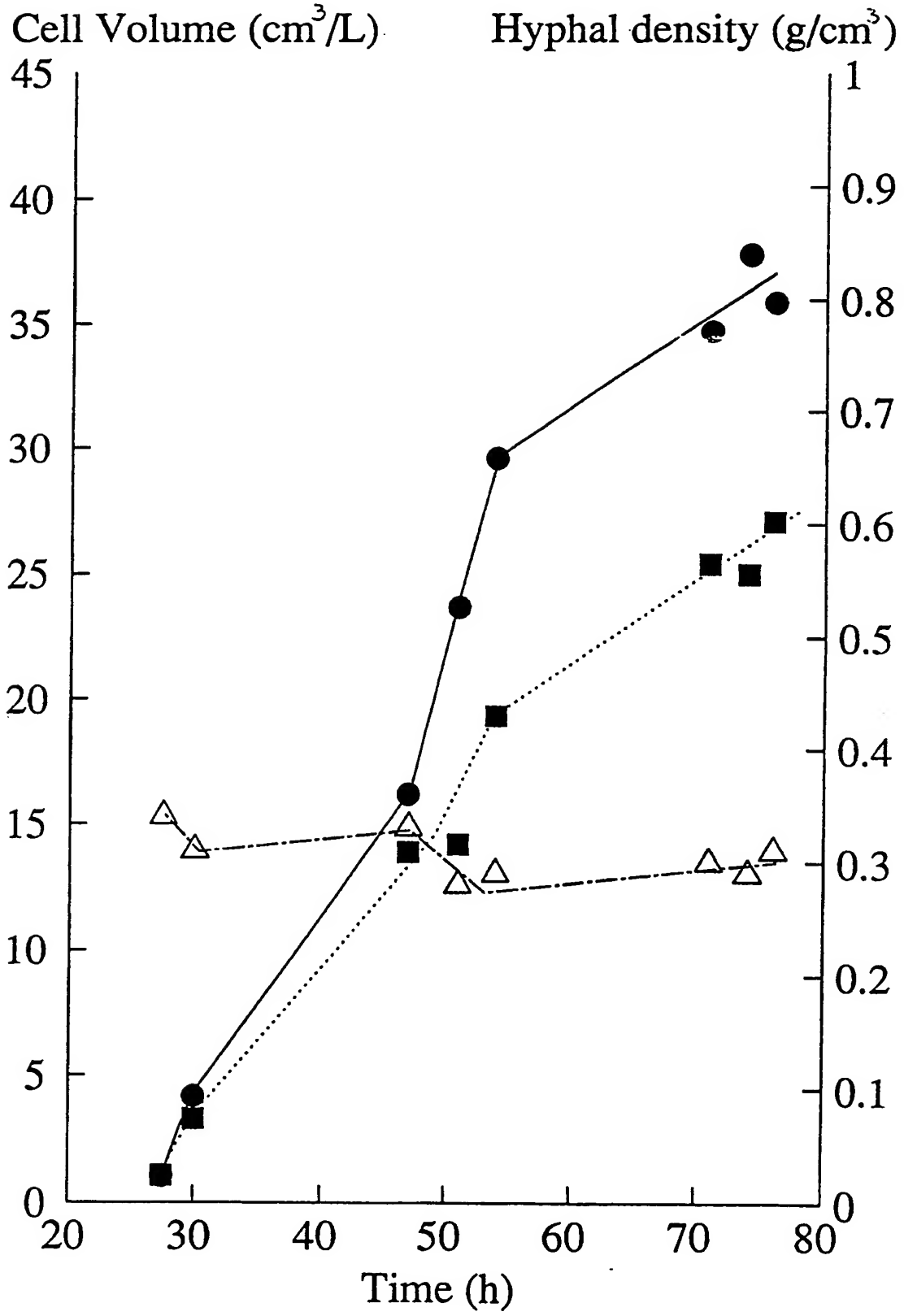

Cell Volume (cm³/L)
Hyphal density (g/cm³)
45
40
35
30
25
20
15
10
5
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
20
30
40
50
60
70
80
Time (h)

FIG 8

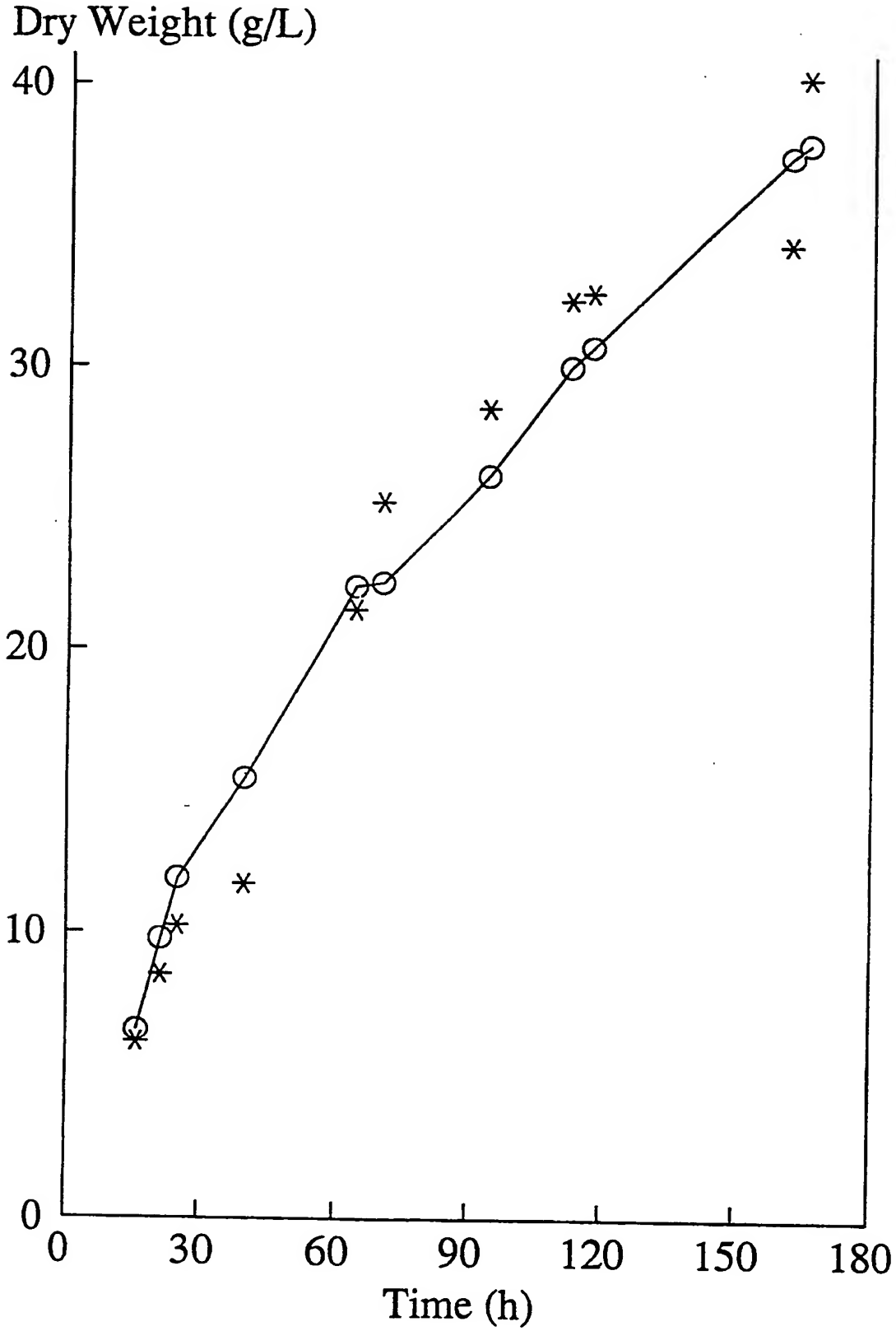
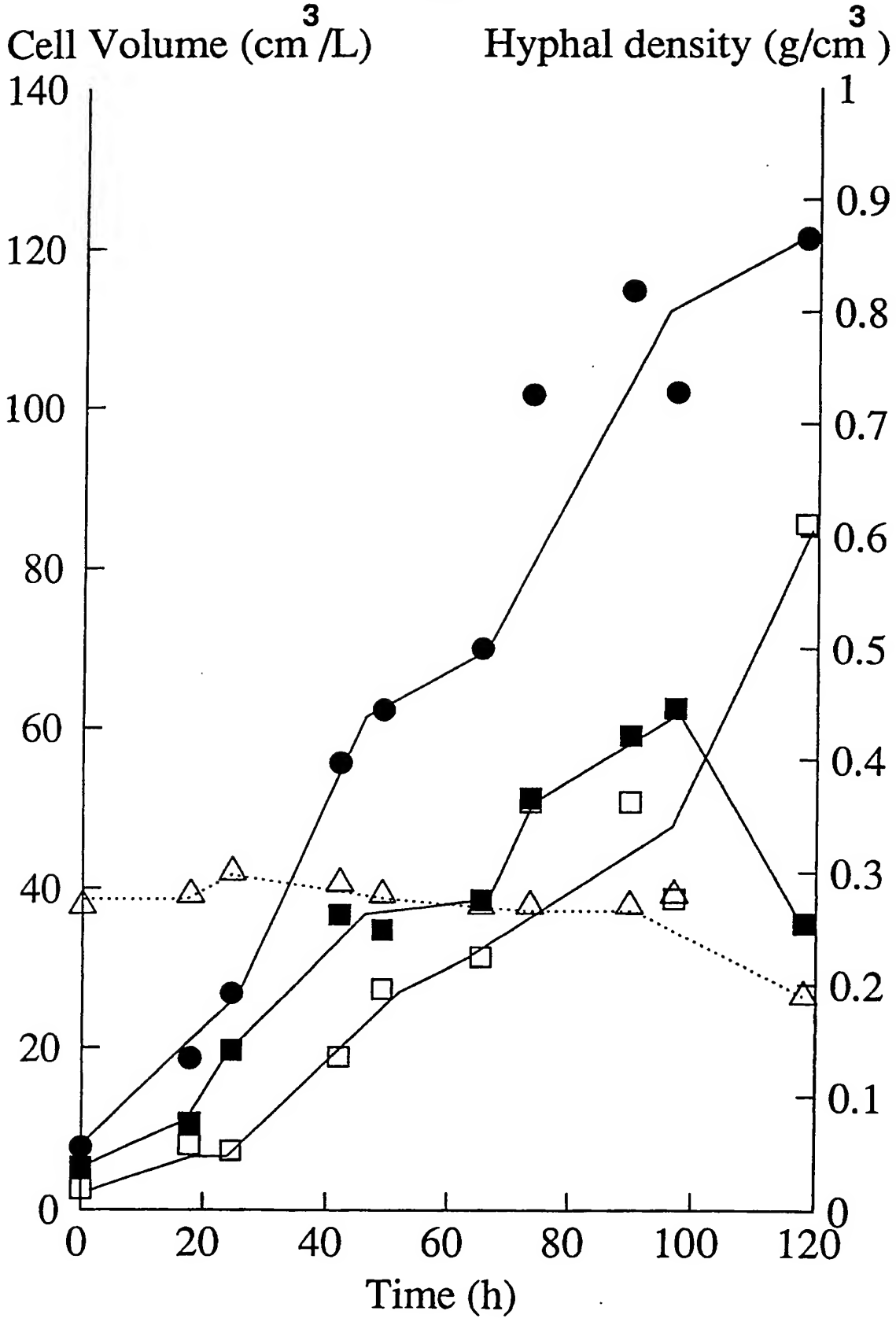


FIG 11.


Cell Volume (cm3/L)
Hyphal density (g/cm3)
140
120
100
80
60
40
20
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Time (h)

"IMPROVEMENTS IN, OR RELATING TO, VOLUME MEASUREMENT OF MICROBIAL ORGANISMS"

The present invention relates to the measurement of cell volume of microbial organisms and in particular to a method which will allow the closer monitoring of cell volume or biomass of microbial organisms, e.g. bacteria, yeast or fungi, in fermentation broths to allow monitoring of the progress of the fermentation process.

Certain industries, e.g. the drug production industry, are increasingly using bio-chemical processes for production, e.g. of antibiotics and other drugs, in particular in what are termed "fermentation processes" in which microbial organisms, e.g. bacteria, yeast or fungi, are grown in a broth to produce, either simultaneously or later, a desired product. As with all industrial processes there is a need to optimise such fermentation processes and to do that it is necessary to be able to measure the growth of the organisms and in particular the change in cell volume or biomass in the broth.

At present there are very few satisfactory techniques for measuring biomass. The simplest technique is to measure the optical density of the broth. In this technique the broth may need to be diluted in order to give an accurate correlation between the optical density and the biomass, but this technique is only suitable for processes where the broth

contains well-dispersed individual cells, e.g. bacteria, in a clear culture medium. It thus has limited applicability. Where the organisms are more complex, e.g. in mycelial form, the organisms frequently become tangled. They can form pellets in such situations having sizes from a fraction of a millimetre to several millimeters across. In such a situation optical density cannot give an accurate measure of biomass, and so the biomass has been measured by centrifuging samples and simply measuring the amount of the solid deposit. Alternatively the broth can be filtered and the solids dried and weighed. However, frequently fermentation broths include undissolved solids as well as the cellular organisms (in some cases due to the use of cheap materials in the interest of increased industrial efficiency) and these techniques are unable to distinguish between such solids and biomass. Furthermore, none of the techniques is capable of characterising the development of the fermentation in terms of the amount in the sample of cytoplasm, vacuolized regions, or lysed regions and such measurements, in addition to basic biomass measurement, would be helpful in enabling closer monitoring and control of the fermentation process.

According to the present invention there is provided a method of determining the cell volume of microbial organisms in a sample including the step of illuminating the sample to provide an image of the sample, distinguishing different regions within the image area by comparison with a

predetermined threshold brightness level, and measuring the area of the distinguished regions within the image area. The invention also provides apparatus for measuring cell volume of cellular organisms in a sample comprising a light source for illuminating the sample, an optical system for imaging the sample and comprising a microscope and camera, a comparator for comparing the brightness of regions of the image to a predetermined threshold level and summing means for summing the total area of regions distinguished with reference to the predetermined threshold level.

The biomass may be calculated by multiplying the measured volume by a density.

The method and apparatus are particularly useful for measuring various filamentous organisms, e.g. filamentous fungi and prokaryotes. Also the method would be advantageous for measuring non-filamentous organisms in broths containing undissolved solids such as industrial fermentations in the presence of Pharmamedia or chalk or molasses.

The predetermined threshold level may be set to include all cellular material or to exclude certain regions such as large vacuoles and lysed regions, or two measurements may be made, one of the total area of cellular material and a second of the area excluding large vacuoles and lysed regions.

Thus with the present invention, measurement of cell volume includes the step of measuring directly the area of cellular material within a sample, from which the cell volume

and thence, if desired, the biomass can be calculated in a number of ways in samples which include organisms in mycelial form. The direct measurement of the area improves the accuracy of the final cell volume/biomass figure.

It is also possible to characterise the development of the organisms in terms of the volume of total cytoplasmic and lysed regions and thus more closely monitor the development of the fermentation process. The quantification of the hyphal regions allows calculation of the rates of change. The proportions of these regions and their rates of change may be related to the rate of product formation and other metabolic parameters such as maintenance coefficients.

The invention may also include the step of eliminating from the measurement regions of the image which satisfy a preset circularity parameter whereby the measurement can be confined to cellular regions of interest excluding undissolved solids which may be present in the sample. The invention can therefore be adapted to operate successfully with fermentation processes in which materials are perhaps of lower quality and thus include much non-cellular material which would confuse a conventional dry mass measurement.

The image may be digitised into a pixel array and areas measured by summing pixels; the pixels may also be assigned to a grey scale on which the threshold or thresholds can be set as desired. The invention may include the further step of excluding from the measurement regions which are darker

than a preset level, or such darker regions and a preset additional area surrounding them. This can assist in excluding areas in which undissolved solid particles overlie the cellular organisms. However, as crossovers between the organisms can also appear dark, the image can be cleaned to eliminate these e.g. by a size threshold (the crossovers are smaller in some cases than the undissolved solids).

Having calculated the area of cellular organisms visible, the volume can be calculated in a variety of ways, for instance by assuming the organisms to be geometrical shapes whose volume can be calculated, possibly with further measurement. For instance, for a mycelial organism the hyphae can be assumed to be solid cylinders and the volume expressed as:-

$$\text{Volume} = \pi \times \text{hyphal diameter} \times \text{area} \times 0.25$$

and so if the diameter of hyphae are measured the volume can be calculated by using the average diameter and the measured area. It is possible to use a mean diameter calculated by measuring a statistically significant sample of hyphae, e.g. approximately 100. The volume can be converted to biomass by multiplying by a density. The values of densities for various organisms are known for both cytoplasmic regions and degenerated regions and so the dry mass of such regions can be calculated.

In the embodiment of the invention below the hyphae diameters are measured manually but automated apparatus can be used to make this measurement.

The setting of the thresholds used to distinguish the organisms from background and to distinguish the different parts of organisms are to some extent system- dependent, for instance they depend on the intensity of the light used for illumination, the sensitivity of the optical system (cameras and microscopes used) and also the type of organism and medium. However, for industrial production, for which this invention is primarily intended, the thresholds need only to be determined on set-up and will not then vary (except for possible drift caused by equipment and materials which can be monitored by regular checking and compensated). The invention can therefore provide frequent automatic monitoring of biomass and automatic monitoring of the development of the fermentation (batch, fed-batch or continuous) by monitoring the volume of cytoplasmic material and lysed material and thus allow better optimization of the process for instance by feedback control.

The invention will be further described by way of non-limitative example with reference to the accompanying drawings in which:-

Fig.1 illustrates regions of hyphal differentiation during a penicillin fermentation;

Fig.2 illustrates the process stages in one embodiment of

the present invention;

Figs.3A-C illustrate processing steps which can be used with the present invention;

Fig.4 is the relationship between the dry cell weight obtained by the conventional filtration method and measurement using the present invention;

Fig.5 is a graph comparing the fermentation time course of dry cell weight obtained by the conventional filtration with measurement using the present invention;

Fig.6 is a graph comparing another fermentation time course of dry cell weight obtained by the conventional filtration with measurement using the present invention;

Fig.7 is a graph illustrating the fermentation time course of the volumes of total and cytoplasmic regions, and also the hyphal density for the fermentation of Fig.6;

Fig. 8 is a graph comparing the fermentation time course of dry cell weight for a fed-batch fermentation measured by the conventional filtration method with measurement using the present invention;

Fig.9 is a graph illustrating the fermentation time course of the volumes of the cytoplasmic and degenerated regions, and also the hyphal density for the fed-batch fermentation;

Fig.10 is a graph comparing the fermentation time course of dry weight for a Lactose/Pharmamedia fermentation measured by the conventional filtration method with measurement using the present invention; and

Fig.11 is a graph illustrating the fermentation time course of cell volumes and hyphal density for the Lactose/Pharmamedia fermentation.

One embodiment of the present invention will be described below followed by a description of particular examples which provides a comparison with conventional methods.

In order to monitor the development of a fermentation using filamentous fungi it is helpful to monitor various stages of cell development. Previous models of cell development have assumed that two cell types are present during fermentation, firstly "healthy" cells completely filled with cytoplasm and secondly degenerated cells from which cytoplasm has been lost. However, a different approach has been taken in applying the present invention which takes into account intermediate stages of cells, these are: firstly the cytoplasmic region which contains all "healthy" cells and partially degenerated cells but excluding large vacuoles, and secondly degenerated regions which contain all of the cells without cytoplasm and the large vacuoles. The sum of the two regions is the total hyphal region and the various regions are illustrated in figure 1.

To measure the cell volume and calculate biomass using the present invention a sample of a fermentation broth is taken for analysis. One method of preparing the sample would be treatment of the sample with preservative to kill the organisms and prevent further development. The sample is

then diluted (the degree of dilution depending on the nature of the broth - many are semi-solids) and a small measured quantity, e.g. four microlitres, is pipetted onto a slide and a fixed area is covered by a cover-slip which is sealed to prevent dehydration. The area beneath the cover slip is then illuminated and imaged using a microscope and video camera connected to and controlled by an image analyzer. The sample preparation including volume used, use of preservative, the slide preparation techniques and microscope magnification can be changed depending on the nature of the sample.

The image area is divided into pixels and each pixel is assigned a greyness level on a grey scale. The thresholds can then be chosen to firstly distinguish between background and all cells present, and secondly to distinguish between cells with a large amount of cytoplasm in them and degenerated cells and vacuolized regions. Thus two different thresholds are set. The number of pixels of each region are then summed for the whole image area. The slide is then moved to image a new area and this process is repeated until the entire area under the cover slip has been processed. This process is illustrated in the flow diagram of Figure 2.

For media which do not contain undissolved solids the processing phases are as shown in Fig.3A where the image is thresholded and any particles removed using, e.g. a circularity parameter. However, most fermentation broths include undissolved solids which should not be included in

the area measurement and it is possible to exclude them. Several methods are available for doing this, one being the use of a circularity parameter (which is 4 π times the area divided by perimeter squared). The parameter can be chosen according to the type of organism used and the solids expected and this method is particularly suitable for filamentous organisms because the more rounded undissolved solids can be clearly distinguished from them. However, the size of the solids can also be used to help distinguish them and, furthermore, it is also possible to distinguish particularly undissolved solids overlying cellular regions of interest by locating their centre (using the fact that it is darker than a certain level) and then locating the perimeter. The processing steps are shown in Figs.3B and 3C. First the image is thresholded to select the dark regions including solids. As crossovers between different hyphae are also dark, these are eliminated from the solids image, e.g. by using the fact that they are smaller than the solids. The positions of the solids on the image are then dilated (see below) to ensure the whole area of the particle is covered. Hyphal fragments can also be removed from the image using further size and circularity parameters. The remaining image is of the solids and is subtracted from the basic threshold hyphal image to leave just the hyphae for measurement.

Once the area of the total cellular region and the cytoplasmic region have been obtained the volume can be

estimated by treating the organisms as a regular geometric body, in the case of hyphae - as a solid cylinder and measuring a diameter of the hyphae visible in the image. This can then be multiplied by a suitable density value to obtain the biomass. The difference between the total cellular region and the cytoplasmic region gives an idea of the amount of material which has degenerated and thus the amount of product produced.

The method above has proved able, in the case of hyphal fungi, to estimate biomass even in the presence of non-dissolved solids of concentration up to 30 grams dry weight per litre and to estimate successfully concentrations of hyphae from 0.03 to 38 grams per litre.

Examples of fermentation and analysis using the present invention together with a comparison with conventional methods will now be given.

Penicillium chrysogenum P-1 was grown as a batch culture using a defined medium or a lactose/Pharmamedia production medium. For the defined medium a 10-L fermenter (Chemap A.G., Mannedorf, Switzerland) with three turbine impellers rotating at 800 rpm (tip speed of $3.35 ms^{-1}$) and an air flow rate of 0.5 vvm was inoculated with a spore inoculum to give an initial value of 10^6 spores mL^{-1}. Two fermentations were carried out using different conditions: fermentation A at 26°C and fermentation B at 30°C. A 5-L fermenter (LH Fermentation Ltd., Maidenhead, UK) containing the

lactose/Pharmamedia medium with three turbine impellers rotating at 950 rpm (tip speed 3.35ms^{-1}) and an air flow rate of 0.5 vvm was inoculated with a 10%(v/v) 54h old mycelial inoculum grown in a non-defined seed medium. The lactose/Pharmamedia production medium contained 30gL^{-1} of undissolved solids post-sterilization measured using the filtered dry weight technique described below.

Penicillium chrysogenum P-2 was also grown in the 5-L fermenter at an impeller speed of 1300 rpm (tip speed 4.6ms^{-1}) and an air flow rate of 0.5 vvm in fed batch culture on the medium described by Mou and Cooney (Biotech. Bioeng., 25, 257 1983). The glucose feed rate was 7.08 mLh^{-1} during the fermentation however the glucose feed concentration was increased after 26h of fermentation time from 485gL^{-1} to 638gL^{-1}. A vegetative inoculum of 1 g dry cell wt L^{-1} was used. The amount of solids present in the medium post-sterilization was approximately 2.5 gL^{-1}. Samples were removed from the fermenters at timed intervals for biomass estimation. To determine the errors due to slide preparation, two samples were used from a Penicillium chrysogenum P-1 shake-flask fermentation. These were taken at 30.5h and 90h after inoculation with spores. The flask contained 10%(v/v) of defined medium and was incubated at 26°C in an orbital shaker (model G25 Incubator shaker, New Brunswick Scientific Ltd., Watford, UK; 200 rpm 5cm throw).

As a comparison with conventional methods a filtered dry

weight measurement was carried out by filtering 5ml of broth through a predried and preweighed glass microfibre filter, Whatman grade GF/A (Whatman Int. Ltd., Maidstone, UK). The sample was washed with 10ml of distilled water before oven drying at 105°C to a constant weight. The results of the measurement are illustrated in the attached drawings.

To estimate the biomass using the present invention samples from the fermentations were mixed with an equal volume of fixative (13mL 40% formaldehyde and 5mL of glacial acetic acid added to 200mL of 50% v/v ethanol). The fixed sample normally was further diluted with fixative to 10 fold, or in the presence of solids to 20 fold. A duplicate set of samples was obtained from a defined medium fermentation. These were combined with a pre-inoculation sample of lactose/Pharmamedia medium and diluted to give a final concentration of undissolved solids equivalent to that of a 20 fold dilution of samples taken directly from the lactose/ Pharmamedia fermentation. During this process the defined media samples were diluted 20 fold.

A 4μL aliquot of each diluted sample was pipetted onto a microscope slide and covered with an 324mm^2 coverslip. The coverslip was sealed to prevent dehydration of the sample during processing. To determine the errors due to slide preparation, 10 slides from the shakeflask sample at 90h were measured and 1 sample was measured from each of 10 repeated dilutions for both shakeflask samples (30.5h and 90h).

For measurement of hyphal regions, a Magiscan MD system (Joyce Loebl Ltd., Gateshead, UK) running general purpose image analysis software, attached to a Polyvar microscope, set for brightfield illumination (Leica Cambridge Ltd., Cambridge, UK) was used. An automatic stage with a 5μm step size was fitted to the microscope, giving automatic X, Y, Z motion and light control. For each sample three measurements were made:- the total area of the mycelia, the area of the cytoplasmic region of the mycelia and the average hyphal diameter.

To measure hyphal area the microscope magnification was set at 100 times and the automatic stage was programmed to give total coverage of the coverslip, to measure all of the mycelia present. The video camera mounted on the microscope sent a video signal of the field of view to the image analysis system where it was digitised in both space and tone producing a pixel array. Each pixel was assigned a greyness level representing the tone of the locality of that pixel in the original image. Once the image was digitised it was then thresholded, i.e. all the pixels of a greyness level in a preset range were treated as being of interest. The thresholding for this system was on a scale of 0 to 63 (64 grey levels). For the total region the thresholding used was 0 to 54. A masking binary image was created in which all the objects of interest had been selected for further processing and thus the background was eliminated. For

measurement of the total area the captured image was thresholded so that all fungal material was included in the binary image whereas for the measurement of the area of the cytoplasmic region the threshold level (for this system levels 0-49) excluded large vacuoles and lysed regions from the binary image. Once thresholded the binary image contained undesirable objects such as media particles as well as hyphae so these were eliminated using a circularity parameter. The circularity for this system excluded particles of circularity greater than 0.35. However for the lactose/Pharmamedia production medium additional processing was required to eliminate the media particles lying on the mycelial aggregates.

There were two categories of particles to be removed:

1. particles entrapped within the mycelial networks
2. particles lying freely outside of the networks

After the image was captured it was thresholded to detect the very dark regions within the media particles by selecting a threshold range of 0 to 26. Crossovers in the mycelial network also often appeared dark and had grey values within this range, therefore it was necessary to eliminate these from the binary image. The thresholded crossovers were less than 20 pixels in area which was less than the size of the dark regions within the media solids so all objects of area less than 25 pixels were removed. As only the dark regions inside the media particles were thresholded it was necessary

to increase the coverage of these regions to encompass the whole of the undissolved particles. A technique called dilation was used to achieve the coverage of the particles. This technique adds pixels to the objects in the binary image. Two layers of pixels were added to the thresholded region. First for every pixel with the value of one is replaced by a cross (3 by 3 pixels) and on the second pass every pixel present, with the value of one is replaced by a 3 by 3 box. This technique enlarged the thresholded pixels to cover the whole media particles as determined by visual observation over many fields of view. However some pieces of mycelia were often still present in the binary image so these were measured and it was determined that their sizes fell into two categories:

1. area between 290 to 800 pixels and circularity 0.2 to 0.8

2. area between 200 to 400 pixels and circularity 0.75 to 1.0.

The above values were used to eliminated the thresholded mycelial fragments from the image. The binary image containing the undissolved solids particles was stored in image memory. The threshold values of brightness, size and circularity may be varied for different systems. The particles may have completely different threshold values from the hyphae and therefore could simply be eliminated by thresholding alone or alternatively may have identical values

to the hyphae. If a similar situation to the lactose/Pharmamedia fermentation arises then expanding the dark thresholded regions to encompass the whole of the particles could be achieved using techniques other than dilation.

The captured image was then thresholded for the total or cytoplasmic regions and the particle elimination parameter of removal of all objects greater than a circularity of 0.35 was applied. The stored binary image containing particles from the mycelial networks was then subtracted from this binary image to eliminate all of the media particles.

The area of the remaining objects, the hyphae, in the treated image was measured by summing all the pixels present in the binary image and applying a scale factor to the sum to calculate the area in square microns. After each field of view the microscope stage moved automatically to the next field until the whole coverslip area had been measured and the data was stored.

In order to estimate the cell volume the hyphae were assumed to be solid cylinders for which the volume is:-

$$\text{Volume} = \pi \times \text{hyphal diameter} \times \text{Area} \times 0.25$$

In this example the average hyphal diameter for each sample was measured manually by touching with a light pen two opposite points on the image of the hyphal wall of randomly

selected hyphae using a microscope magnification of 400 times. The inter-point distance was then calculated by the image analyzer and stored in a data file. For each sample the process was repeated at least 100 times using new positions on the same and on different hyphae.

The volume was calculated for the total hyphae, the cytoplasmic region and the degenerated region was calculated as the difference between them. Taking into account the sample dilution factor the result was converted into cm^3 of cell volume per Litre of fermentation broth.

Once the volumes of the regions had been measured they were converted into dry weight estimations. The values of hyphal densities (g dry weight/cm^3 of cell volume) for Penicillium chrysogenum are available in literature, for cytoplasmic regions it is $0.35g/cm^3$ and for degenerated regions $0.18g/cm^3$. Therefore the volumes can be converted to biomass:-

Dry Weight (g/L) = (volume of cytoplasmic region (cm^3/L) x $0.35(g/cm^3)$)

+ (volume of degenerated region (cm^3/L) x $0.18(g/cm^3)$)

The results are shown in the attached Figures together with a comparison with the conventional measurements.

Statistical analysis, using one way analysis of variance at the 95% confidence level, showed that there were no significant differences in the distribution of mycelial material on the slide for both the repeated dilutions and

slide preparations. For the sample at 90h the % error of pipetting material onto the slide, at the 95% confidence level, was 8.3% and for the dilutions it was 7.3%. For the young cells (30.5h) the error of dilution was 4%. The percentage of material present in the form of aggregates was 76% for the sample at 30.5h and 96% for the sample at 90h.

The relationship between the dry cell weight estimated by the present invention and measured by conventional filtration is shown in Figure 4 for the pooled samples of a number of fermentations examples of which are discussed below. The relationship shown is the line of best fit through the data which has a correlation coefficient of 0.99 at the (95% confidence level). This shows the good agreement between the dry cell weight estimated by the present invention and the measured dry weight using the conventional method.

Figures 5 and 6 show different time courses of biomass for two fermentations, A and B, in the defined media measured by the conventional method (O) and, by the present invention (X). In Figure 4 the spores germinated and growth occurred until 45h when the hyphae began to undergo lysis. In the second fermentation (Fig.5) a different profile with slower growth was obtained. The symbol □ shows the result of estimation using the present invention with solids present in the sample (see below). For both fermentations the estimate of biomass from the image analysis methodology closely matches the values obtained from the conventional method. In

fermentation A (Fig. 4) after 62h the dry weight decreased as lysis occurred and the technique using the present invention was able to follow this event successfully.

The time courses of the volumes of the total hyphae and the cytoplasmic region, measured by the present invention are shown in Figure 7 for fermentation B. The total hyphal (●) and the cytoplasmic (■) volumes were equivalent until 30h after spore inoculation then vacuolization was detected resulting in the divergence of the profiles. The average hyphal density (Δ) is also shown for the time course, initially this was about 0.35g/cm^3 and then, as the profiles of volume diverge, dropped to approximately 0.31g/cm^3 for the remainder of the fermentation. The measurements of hyphal diameters for fermentation B ranged from 4.3 to 5.2μm. Differentiation of the various regions in this way is not possible using the conventional technique. Figure 5 also shows the dry weight profile obtained when lactose/Pharmamedia medium was added to samples of fermentation B. The biomass measurements obtained using the present invention were unaffected by the presence of the non-dissolved medium solids.

The biomass time course of the fed-batch fermentation is shown in Figure 8, once again the estimate using the present invention (X) of dry weight matched the measurement obtained by the conventional method (O). The fed batch fermentation initially contained a small amount of undissolved solids in

the media but there is no evidence of their presence in the dry cell weight profiles. Therefore considering that the first sample was at 16.25h after inoculation it is likely that the solids had been broken down and were only present, if at all, in insignificant quantities. The total cell volume (●) and hyphal density (△) profiles for this fermentation are shown in Figure 9. The total cell volume increased steadily throughout the time course. The volume of the cytoplasmic region (■) however increased rapidly until 64h after which the rate of increase of volume decreased. The mean hyphal diameters for the samples ranged from 6.8 to 5.2μm.

The biomass time course for the lactose/Pharmamedia fermentation, measured by the conventional method (O) and the present invention (X) is shown in Figure 10. Until 65h the solids masked the hyphal growth in the measurements by filtration, then the dry weight increased until 97h, after which it decreased slightly. The biomass estimated by image analysis increased steadily after inoculation until 90h when it decreased rapidly. Figure 11 shows the time course of the volumes of the total hyphae (●), the cytoplasmic region (■), the degenerated region (□) and the hyphal density (△) for the lactose/ Pharmamedia fermentation. The total hyphae increased steadily until 90h when it reached a plateau at approximately 115cm^3. The volume of the cytoplasmic region also increased until 97h after which it dropped rapidly. The

mean hyphal diameters for the samples from the lactose/Pharmamedia fermentation ranged from 4 to 6μm. The conventional technique could not give any information regarding the cytoplasmic or degenerated regions, only total biomass in the absence of solids.

Thus, the measurements of the hyphal volumes followed the hyphal growth during the lactose/Pharmamedia fermentation (Fig.11). The inoculum was mycelial so vacuolization had already occurred and the cytoplasmic and total hyphal volumes already had different values. The profiles continued to diverge throughout the fermentation. When cell death and lysis started after 97h the volume of the cytoplasmic region decreased dramatically but the total cell volume remained fairly constant as the cells walls tend to be stable. These events are also indicated in the hyphal density which decreased at the onset of cell death and lysis. The measurement by the technique of mycelia in the high non-dissolved solids concentrations during the lactose/ Pharmamedia fermentation showed the effectiveness of measurement using the present invention in avoiding measurement of the solids particles to give a direct measurement of the volume of the hyphal regions.

While the repeated dilutions and slide preparations showed that the major error in the method was due to the placement of the 4μL sample onto the slide (as the dilution error was masked by the error of pipetting onto the slide and the sample

at 90h showed a larger error than the one at 30.5h due to the different morphologies and dispersions of the two samples) the error on the biomass estimations due to sample preparation using the present invention is approximately ±8.4% which compares well with the error of measurement of the conventional method at the 95% confidence limit of ±12 to 2% respectively with the error being greatest at low dry weights.

In the examples above the technique was used to estimate biomass in fermentation samples ranging from 0.68 to 38gL^{-1} before dilution. If dilution is taken into account the minimum cell concentration used was 0.034gL^{-1} and so the resolution can be increased as the sample can simply be diluted. The performance was also not affected by the presence of up to 30gL^{-1} of non dissolved solids in the media (Fig.6 and 10) and provided measurement of the total, cytoplasmic and degenerated hyphal cell volumes which are very helpful in fermentation studies.

While sampling and diameter measurement were carried out manually in the above example, these can be automated to provide a completely automated process for providing rapid direct measurement of hyphal volumes and estimates of biomass, even in industrial fermentations with high concentration of non-dissolved solids. The technique can therefore be used on-line with the fermentation conditions being controlled according to the results, e.g. completely

automatically by including means for taking and processing samples and control means for controlling fermentation conditions according to the results.

C L A I M S

1. A method of determining the cell volume of microbial organisms in a sample including the step of illuminating the sample to provide an image of the sample, distinguishing different regions within the image area by comparison with a predetermined threshold brightness level, and measuring the area of the distinguished regions within the image area.

2. A method according to claim 1 wherein the predetermined level is set to include all cellular material.

3. A method according to claim 1 wherein the predetermined level is set to exclude large vacuoles and lysed regions.

4. A method according to claim 1, 2 or 3 including the step of eliminating solid particles from the image.

5. A method according to claim 1, 2, 3 or 4 further including the step of eliminating from the measurement regions of the image which satisfy a preset circularity parameter whereby the measurement can be confined to cellular regions of interest.

6. A method according to any one of the preceding claims including the step of digitizing the image into a pixel array.

7. A method according to claim 6 wherein the pixels are assigned to a grey scale and the threshold is set on the

scale.

8. A method according to any one of the preceding claims wherein regions darker than a preset level are detected and excluded from the area measurement.

9. A method according to claim 8 wherein said darker regions and a preset additional area surrounding them are excluded from the area measurement.

10. A method according to any one of the preceding claims further including multiplying the measured area by a width parameter to derive a measurement representative of cell volume.

11. Apparatus for determining cell volume of microbial organisms in a sample comprising a light source for illuminating the sample, an optical system for imaging the sample and comprising a microscope and camera, a comparator for comparing the brightness of regions of the image to a predetermined threshold level and summing means for summing the total area of regions distinguished with reference to the predetermined threshold level.

12. Apparatus according to claim 11 further including setting means for setting a plurality of different thresholds whereby the summing means sums areas with respect to the different thresholds.

13. A method of measuring cell volume of microbial organisms substantially as hereinbefore described with reference to and as illustrated in the accompanying drawings.

14. Apparatus for measuring cell volume constructed and arranged to operate substantially as hereinbefore described with reference to and as illustrated in the accompanying drawings.

15. A method of determining biomass in a sample comprising measuring the cell volume according to the method of any one of claims 1 to 10 or 13 multiplying the cell volume by a density parameter to obtain a biomass-representative measurement.

16. A method of monitoring the progress of a fermentation process to optimize the process including the step of measuring the cell volume according to any one of claims 1 to 10 or 13.

17. The use of apparatus according to claim 11, 12 or 14 to measure all volume in a fermentation broth.

Patents Act 1977
. ɹminer's report to the Comptroller under Section 17 (The Search Report)

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Relevant Technical fields

(i) UK Cl (Edition K) G1A AAJ

(ii) Int Cl (Edition 5) G01N

Search Examiner

S J PROBERT

Databases (see over)

(i) UK Patent Office

(ii)

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Documents considered relevant following a sea... n respect of claims 1-17

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
X	GB 2152660 A (SII (FRANCE))	1,2,6, 7,11
X	GB 1519704 (CARL ZEISS-STIFTUNG)	1
X	GB 1385679 (IMAGE ANALYSING COMPUTERS)	1,2,11
X	WO 81/03080 (RUSH PRESBYTERIAN)	1,11

Category	Identity of document and relevant passages	Relevant to claim(s)

Categories of documents

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